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TITLE: Customized Fabrication of Osteochondral Tissue for Articular Joint Surface Repair

PRINCIPAL INVESTIGATOR: Rocky S. Tuan, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213-2303

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Rocky S. Tuan, Hang Lin, Aaron Sun, Peter Alexander

E-Mail: rst13@pitt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
450 Technology Drive,
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Osteoarthritis (OA) is a chronic degenerative disease of the articular joint that involves more than just the destruction of the articular cartilage – it is a disease of the cartilage, bone and surrounding soft tissue that disables 9-10% of the US population. In the US military, combat and non-combat injuries experienced during deployments, training and generally active life-style can cause traumatic mechanical destruction or progressive biological degeneration of the articular cartilage. It has been reported that OA is the most frequent cause of disability in the armed forces. There are currently no effective therapies for OA. A regenerative surgical approach that aims for biological resurfacing of the joint is thus needed. In this study, we will construct adipose stem cell-laden scaffolds loaded with microparticles, designed to provide temporospatially specific differentiation cues for chondrogenesis and osteogenesis, by the 3D printing method of projection stereolithography (PSL). We further test the applicability of these novel osteochondral tissues for articular cartilage repair in rabbit model, using medical imaging-guided PSL. Such an approach may be adapted for the development of a single-step, point-of-care procedure for clinical cartilage repair. If successful, the technology being developed here could accelerate the rate of recovery and therefore shorten the time away from active duty status and the duration of rehabilitation, while allowing full recovery of joint function and minimizing the impact on military medical care costs.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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TABLE OF CONTENTS

1. INTRODUCTION:

Osteoarthritis (OA) is a chronic degenerative disease of the articular joint that involves more than just the destruction of the articular cartilage – it is a disease of the cartilage, bone and surrounding soft tissue that disables 9-10% of the US population. In the US military, combat and non-combat injuries experienced during deployments, training and generally active life-style can cause traumatic mechanical destruction or progressive biological degeneration of the articular cartilage. It has been reported that OA is the most frequent cause of disability in the armed forces. There are currently no effective therapies for OA. A regenerative surgical approach that aims for biological resurfacing of the joint is thus needed.
In this study, we will construct adipose stem cell-laden scaffolds loaded with microparticles, designed to provide temporospatially specific differentiation cues for chondrogenesis and osteogenesis, by the 3D printing method of projection stereolithography (PSL). We further test the applicability of these novel osteochondral tissues for articular cartilage repair in rabbit model, using medical imaging-guided PSL. Such an approach may be adapted for the development of a single-step, point-of-care procedure for clinical cartilage repair. If successful, the technology being developed here could accelerate the rate of recovery and therefore shorten the time away from active duty status and the duration of rehabilitation, while allowing full recovery of joint function and minimizing the impact on military medical care costs.

2. KEYWORDS:

Post Traumatic Osteoarthritis, Injury, Articular Cartilage, tissue-engineered, 3D printing, Osteochondral constructs.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Goals:
1. Convert micro-computed tomography (micro-CT) images of bone and cartilage of the joint into 3D .stl files to yield a virtual, structural replica of the articular joint to be used for projection stereolithography (PSL).
2. Fabricate bony and cartilage tissues using different biomaterials using PSL.
3. Implant pre-differentiated or undifferentiated osteochondral constructs for focal cartilage lesion repair and assess their reparative efficacy.
4. Test the applicability of tissue engineered osteochondral construct for repair of post-traumatic OA articular cartilage, using micro-CT imaging guided PSL fabrication.

What was accomplished under these goals?

All the results are presented in the end of the report.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

1. Apply PSL to fabricate and assemble osteochondral tissues, in which cartilage side will be composed with hASCs and TGF-β3/BMP-6 loaded PDLLA-PEG hydrogel and bone side will be composed with BMP-2 loaded porous PCL-Heparin scaffolds.
2. Implant pre-differentiated or undifferentiated osteochondral constructs for focal cartilage lesion repair and assess their reparative efficacy.
3. Testing efficacy of tissue engineered osteochondral construct for repair of post-traumatic OA articular cartilage by repairing focal defects in animals, *in vivo*.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

The work for this study will not be completed by 8/24/16 due to:

1. The animal protocol was approved in October 2015, which has been 8 months behind the schedule listed on organically Statement of Work.
2. Space and surgical team time constraints delayed surgery until August 2016.
3. As proposed, we plan to scan condyles from 10 rabbits in order to create a database of rabbit condyle structure. The μCT scanner that we are using for this project is also shared in the Department. Therefore, the progress is slow. We have contacted with the DLAR at Pitt about the use of a μCT scanner owned by BRDGII animal facility, and will start to scan with this machine in the beginning of June 2016.

During the approved no cost extension period (8/25/16 – 8/24/17), we will perform the following work

1. Implantation of optimally undifferentiated osteochondral constructs for focal lesion repair
2. Implantation of optimally pre-differentiated osteochondral construct for articular surface repair
3. Test efficacy of tissue engineered osteochondral construct for repair of post-traumatic OA articular cartilage

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

Sun AX, Lin H, Beck AM, Kilroy EJ, Tuan RS. (2015) Projection Stereolithographic Fabrication of Human Adipose Stem Cell-incorporated Biodegradable Scaffolds for Cartilage Tissue Engineering. Frontiers in Bioengineering and Biotechnology.

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers, and presentations.

(1) Live Cell-scaffold Printing using Biodegradable PDLLA-PEG/ Hyaluronic Acid Copolymer for Cartilage Tissue Engineering H. Lin, A. Sun, A. Beck, E. Kilroy, R. Tuan; 2015 4th TERMIS World Congress Boston, Massachusetts.
(2) Medical Imaging-guided Additive Manufacturing of Human Osteochondral Tissues H. Lin, E. Kilroy, P. Alexander, R. Tuan; 2015 4th TERMIS World Congress Boston, Massachusetts
(3) Medical Imaging-guided Additive Manufacturing of Human Osteochondral Tissues H. Lin, E. Kilroy, P. Alexander, R. Tuan; 2015 Military Health System Research symposium, Ft. Lauderdale, FL

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	*Rocky S. Tuan, PhD*
Project Role:	*Principal Investigator (PI)*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1.20 Person Months*
Contribution to Project:	*He will have direct responsibility for the overall design and conduct of the study, oversight of data analysis, and preparation of research manuscripts and research reports. Dr. Tuan will supervise the day-to-day research activities of all personnel.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217***)**

Name:	*Peter G. Alexander, PhD*
Project Role:	*Data Analyst Scientist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4.80 Person Months*
Contribution to Project:	*In addition to contributing to the overall study design, he will be directly responsible for day-to-day organization of the bench-top research associated with all proposed tasks. He will also play a leading role in the implantation, in vivo imaging and end-point assessment of the constructs in Task 3A & B. He will also be responsible for training the Postdoctoral Associates on all assays proposed, and working with them to complete theses assays.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217***)**

Name:	*Hang Lin, Phd.*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6.00 Person Months*
Contribution to Project:	*He will participate in all activities that require cell entrapment in 3-D matrices, scaffold culture and testing. Thus, he will be involved in optimization of 3D scaffold preparation by PSL, culture of scaffolds, and mechanical and biological testing of the scaffolds.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217***)**
Name:	*Aaron Sun*
Project Role:	*Graduate Student Researcher*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1.80 Person Months*
Contribution to Project:	*Mr. Sun will be testing the biomechanical aspects of the different materials used for the osteochondral construct and also the biocompatibility and regenerative capacity of the printed tissue with the stem cells used. He will also be assisting with the 3D printing of the material.*
Funding Support:	*Supported by internal University Discretionary Funds*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Rocky Tuan	**Closed** DoD grant W81XWH-08-2-0032 "Regenerative Repair of Traumatic Articular Cartilage Injuries: Point-of-Care Application of Mesenchymal Stem Cells and Chondrocytes" Role: PI Effort: 5% Date: 11/1/2012 – 9/28/2015 No impact on this project (W81XWH-14-1-0217)
Rocky Tuan	**Closed** RiMED grant No Identifier "RiMED Fellows Program" Role: Mentor/PI

	Effort:3.88% Date: 6/29/2011 – 5/16/2016 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Closed** DoD grant W81XWH-13-2-0030 "Development of Novel Bioartificial Ligament Using Autologous Biological Scaffold and Cells" Role: Co-Investigator (PI: Dr. Hyun Joon Paek, Tissue Genesis Inc.) Effort: 5% Date: 8/1/13 – 4/28/16 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** DOD grant W81XWH-15-1-0600 "Adult Stem Cell-Based Neurotrophic Conduit Enhancement of Peripheral Nerve Repair" Role: PI Effort: 15% Date: 12/1/14 – 11/30/18 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** CASIS Award# Agrmt# GA-2016-236 A Microphysiological 3D Organotypic Culture System for Studying Degradation and Repair of Composite Skeletal Tissues in a Microgravity Environment Role: Co-PI Effort: 5% Date: 4/30/16 - 12/31/17 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** IFER Grant No Identifier "Application of Human iPSC-derived Mesenchymal Progenitor Cells to Develop Osteochondral Microtissues for Osteoarthritis Drug Testing" Role: Mentor Effort: 0% Date: 12/31/15 - 12/31/16 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** NIH Grant 1R01 GM115444-01A "Why Don't Lizards Regenerate Perfect Tails Like Salamanders?" Role: Co-Investigator (PI: Thomas Lozito) Effort: 15% Date: 5/1/16 - 4/30/21 No impact on this project (*W81XWH-14-1-0217)*

Peter G. Alexander	**Closed** DoD grant W81XWH-08-2-0032 "Regenerative Repair of Traumatic Articular Cartilage Injuries: Point-of-Care Application of Mesenchymal Stem Cells and Chondrocytes" Role: Co-Investigator Effort: 15% Date: 11/1/2012 – 9/28/2015 No impact on this project (W81XWH-14-1-0217)

Peter G. Alexander	**Received** DOD grant W81XWH-15-1-0600 "Adult Stem Cell-Based Neurotrophic Conduit Enhancement of Peripheral Nerve Repair" Role: Co-Investigator Effort: 20% Date: 12/1/14 – 11/30/18 No impact on this project (*W81XWH-14-1-0217)*
Peter G. Alexander	**Received** CASIS Award# Agrmt# GA-2016-236 A Microphysiological 3D Organotypic Culture System for Studying Degradation and Repair of Composite Skeletal Tissues in a Microgravity Environment Role: Co-Investigator Effort: 15% Date: 4/30/16 - 12/31/17 No impact on this project (*W81XWH-14-1-0217)*

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is

acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

What was accomplished under these goals?

The results done in 2015 has been reported in 2015 annual report. Here, we only listed the results conducted in the last year. It will be helpful to refer 2015 report for better understanding the data in this report.

1. Currently, different cell densities have been used in conducting adipose-derived stem cell(ADSC) chondrogenesis. To determine an optimal cell density, we have performed a side-by-side comparison test. We seeded 4 million (M)/ml, 8M/ml, 20M/ml and 50M/ml stem cells within 30% PDLLA-PEG scaffolds and cultured in chondrogenic medium for 4 weeks. As shown in Figure 1, 20M/ml and 50M/ml groups produced equal amount of GAG, which is significantly higher than other 2 groups. We then examined the GAG production efficiency by diving the GAG with total cell number (Figure 2), indicating 20M/ml is an optimal cell density for chondrogenesis.

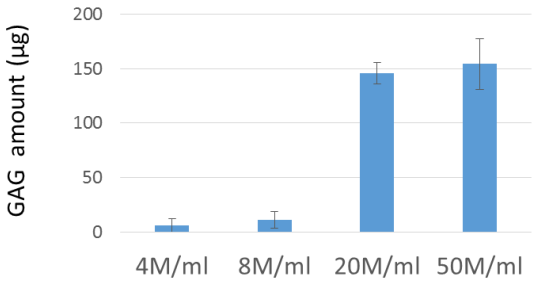

Figure 1. GAG assay after 4 weeks of chondroinduction

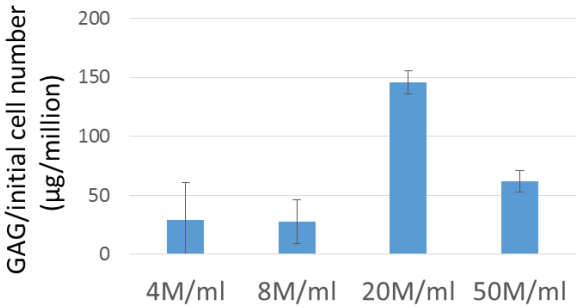

Figure 2. Comparison of GAG production efficiency from different cell density.

2. As shown in 2015 annual report, to induce *in situ* chondrogenesis within hydrogel, we directly loaded TGFβ3 protein into hydrogel with cells. Results showed that 2 μg/ml TGF-β3 incorporation was able to promote cell-laden construct produce GAG with highest efficiency without TGF-β3 supplement. More importantly, the total GAG amount is comparable to those from constructs cultured in TGF-β3-containing medium. We then tested the release profile of TGF-β3 from PDLLA-PEG/Gelatin constructs in order to understand the mechanism. 2 μg/ml TGF-β3 was encapsulated into PDLLA-PEG/Gelatin and soak in PBS. PBS were changed every 3 days up to 28 days. TGF-β3 released to PBS were quantitated using an ELISA kit (Figure 3). As shown in Figure 4, both hydrogels have capacity in controlling the release of TGF-β3. In comparison with pure PDLLA-PEG, gelatin encapsulated hydrogel showed less TGF-β3 release in the first 3 days and more afterwards, indicating a more controlled release manner. This experiment strongly support the biological results we observed. In summary, TGF-β3 loaded within PDLLA-PEG/Gelatin hydrogel is slowly released up to 20 days, which continuously stimulates chondrogenesis and directs cartilage formation.

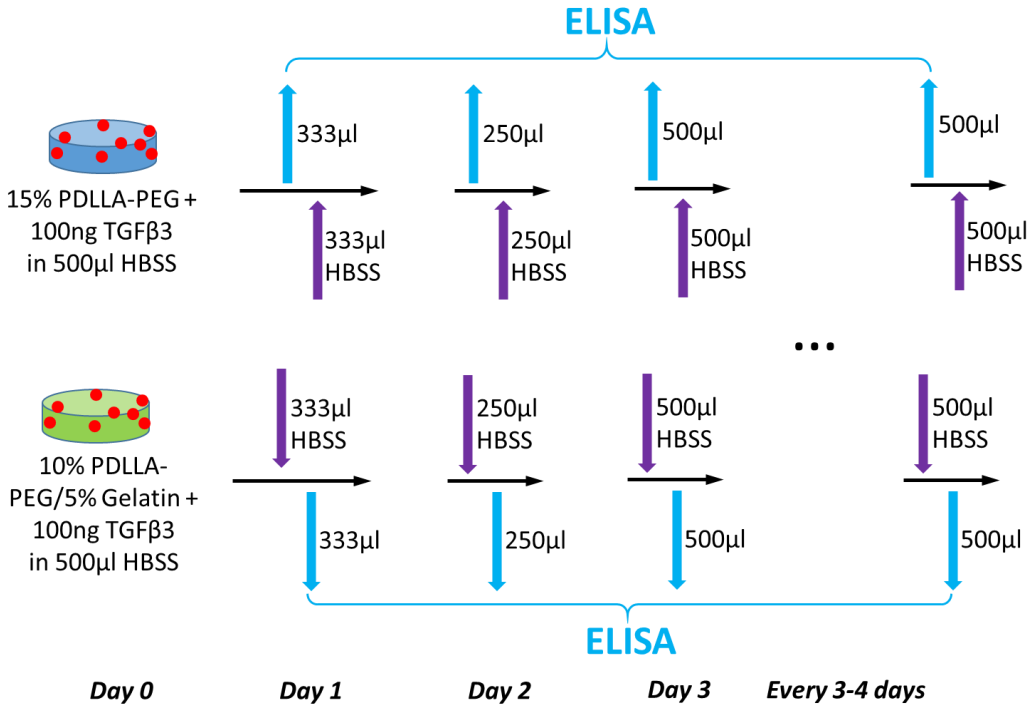

Figure 3. Experimental design of testing the TGF-β3 release from PDLLA-PEG/Gelatin or PDLLA-PEG constructs.

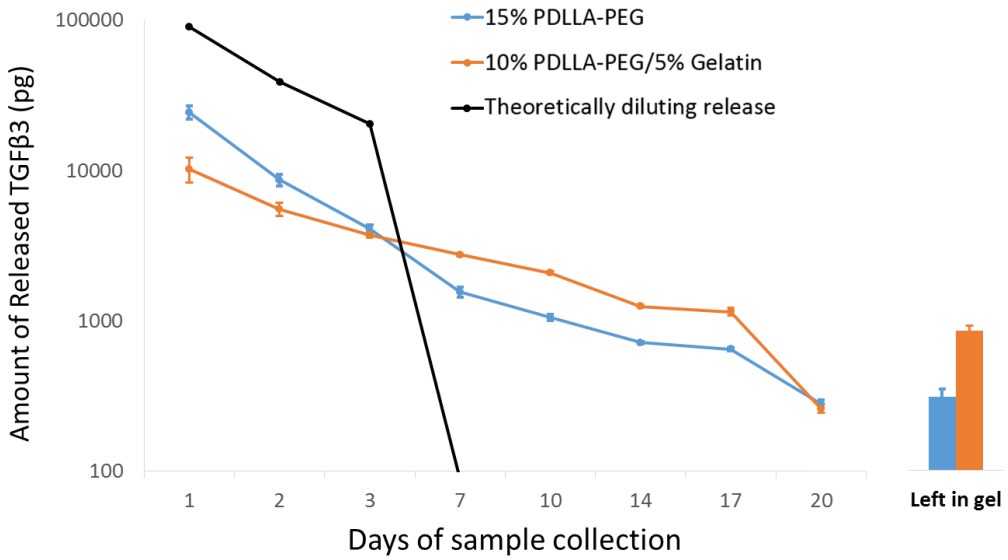

Figure 4. TGF-β3 release from PDLLA-PEG or PDLLA-PEG/Gelatin hydrogel.

3. Currently, PDLLA-PEG is a suitable material to complete this study. We would like to further explode other materials in order to achieve better chondrogenesis, such as higher mechanical property. We developed 2 new materials: (1) PDLLA-PEG with lower molecular weight PEG (PDLLA-LMW-PEG) and (2) poly-L-lactide (PLLA) –low molecular weight PEG (PLLA-LMW PEG). We then seeded stem cells within these 3 scaffolds with different material density at 20%, 25% and 30% (w/v). The cell density was 20M/ml, which has been shown to be optimal for chondrogensis before.

Figure 5 shows the mechanical properties of PDLLA-PEG 4000, PDLLA-PEG 1000, and PLLA-PEG 1000 over 28 days in HBSS. Due to the presence of ester bonds between lactide molecules and PEG, the polymers are expected to degrade through hydrolytic cleavage. The compressive moduli of the scaffolds degrade steadily over time, with a $p<0.001$ between each time point within each group, except for between days 21 and 28 where degradation started slowing down. Statistically significant differences were found in group and concentration effects too, with $p<0.001$ for both group and concentration effects with regard to mechanical strength. PDLLA-PEG 1000 and PLLA-PEG 1000 are significantly stronger than PDLLA-PEG 4000, as evidenced by a 3-4 fold higher compressive modulus at all time points. In addition, the low molecular weight scaffolds did not swell in HBSS which may have contributed to their strength.

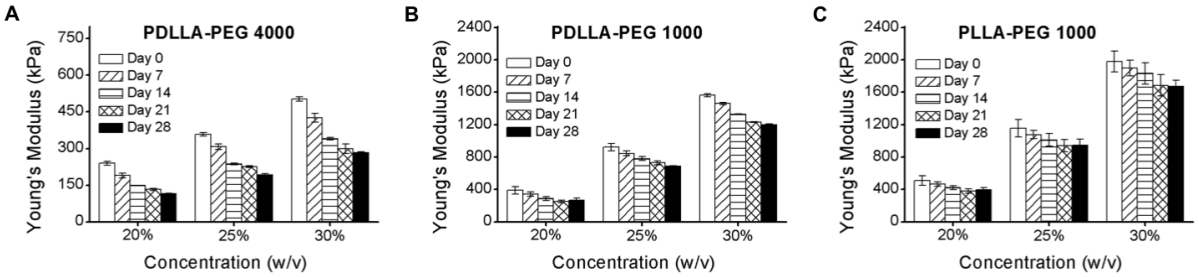

Figure 5. Mechanical properties and degradation of polymers at different concentrations and timepoints. (A-C) Statistical significance of $p<0.001$ is achieved between all subsequent timepoints at each concentration for each material, except for between days 21 and 28 where $p > 0.5$. In addition, the main effects of material type and material concentration are significant at $p<0.001$ based on two-way independent ANOVA.

We then seeded stem cells within these 3 scaffolds with different material density at 20%, 25% and 30% (w/v). The cell density was 20M/ml, which has been shown to be optimal for chondrogensis before. Cell viability was determined at day 1 and day 7 for PDLLA-PEG 1000 and PLLA-PEG 1000 (Figure 6). Viability remained high between the post-fabrication period and day 7 with cell viability >85% in all groups at day 7. The viability did not change significantly between the different concentrations of polymer, indicating adequate nutrient diffusion at all concentrations and biocompatibility of the materials.

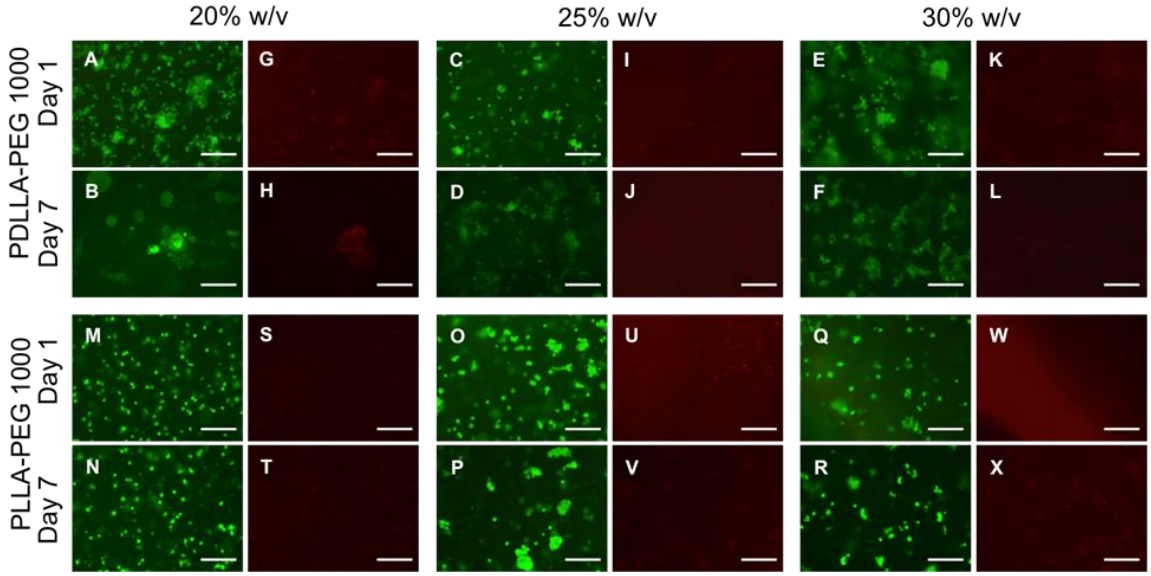

Figure 6. Cell viability in hydrogel constructs. (A-F, M-R) Calcein-AM staining (green, live cells) and (G-L, S-X) EthD-1 staining (red, dead cells) in scaffold following fabrication at days 1 and 7 across 20%, 25%, and 30% w/v polymer concentrations. Cell viability at day 7 was >85% in all groups based on green count/ total count. Scale bar = 150 μm.

Mechanical properties of cell-loaded PDLLA and PLLA-PEG 1000 were significantly higher at all concentrations and time points than corresponding PDLLA-PEG 4000 ($p<0.001$) (Figure 7A). Again, mechanical strength was not maintained over the 28-day culture period. DNA content was

significantly higher in the PDLLA-PEG 4000 groups across the 3 concentrations than in the other low molecular weight groups ($p<0.001$) most likely due to the swelling of the scaffold allowing for more space for cells to proliferate (Figure 7B). Due in part to this higher cell number, there was a significant group effect for PDLLA-PEG 4000 vs. PDLLA and PLLA-PEG 1000 in both GAG and hydroxyproline levels ($p<0.005$) (Figure 7C, 7E). However, when DNA was accounted for there was no significant difference between GAG/DNA and hydroxyproline/DNA in the PDLLA-PEG 4000 vs PDLLA-PEG 1000 groups ($p>0.5$ for both)(Figure 7D,7F). In all measures of ECM production, PLLA-PEG 1000 was significantly lower than either of the other two groups ($p<0.001$)(Figures 7C-7F). In addition, there was a significant concentration effect for GAG/DNA and GAG/construct, with $p<0.001$ for 20% vs. 25% and 20% vs. 30% and $p=0.02$, $p=0.06$ for 25% vs 30% in GAG/construct and GAG/DNA, respectively (Figures 7C, 7D).

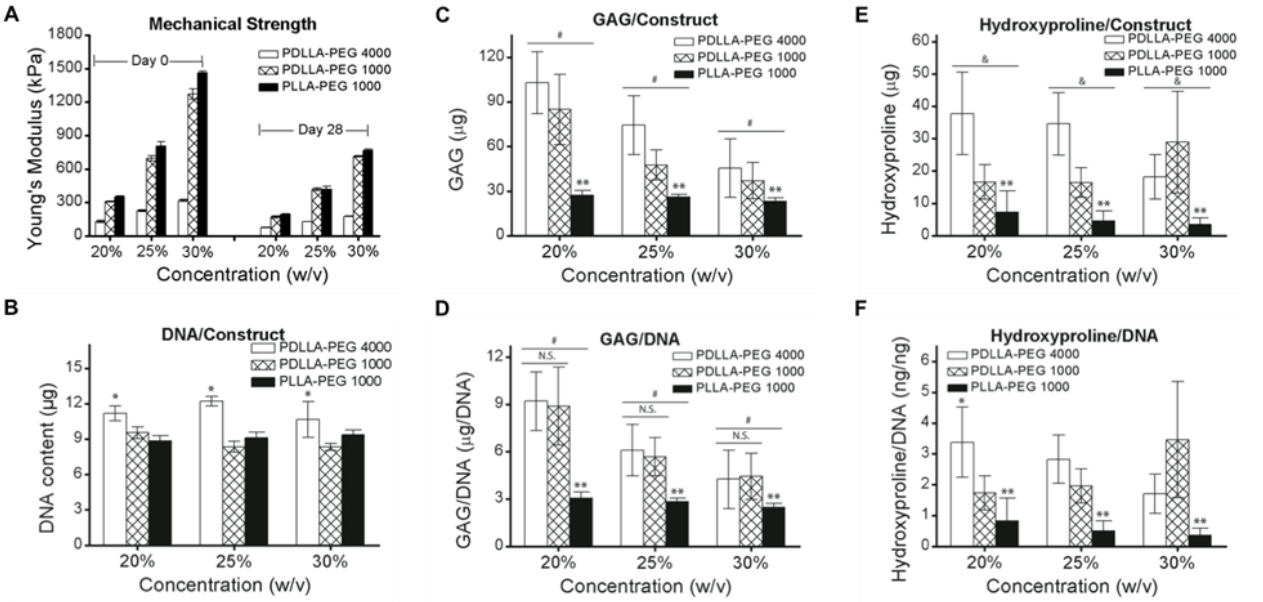

*Figure 7. Effect of material concentration and stiffness on ECM deposition. (A) Mechanical strength post fabrication and after 4 weeks of culture. Mechanical properties of PDLLA and PLLA-PEG 1000 were significantly higher at all concentrations and timepoints than corresponding PDLLA-PEG 4000 ($p<0.001$). (B) Cell number measured by DNA content in constructs. PDLLA-PEG 4000 had significantly higher DNA content likely due to degradation-induced volume expansion. (C,E) Total ECM deposition measured by GAG and Hydroxyproline production per construct, respectively. There exists a significant effect between material concentration and GAG production. (D, F) ECM deposition normalized to DNA content. No significant difference is found between PDLLA-PEG 4000 and 1000 for GAG production despite their significantly different stiffnesses. * $p<0.001$ as compared to other materials at same concentration. ** $p<0.001$ for main effect of material as compared to others. # $p<0.05$ for main effect of material concentration between concentrations and $p<0.005$ for main effect of PDLLA-PEG 4000 vs. other groups. & $p<0.005$ for main effect of PDLLA-PEG 4000 vs. other groups. All effects were determined by Tukey's HSD post-hoc testing following two-way independent ANOVA analysis.*

Chondrogenic gene expression for the different concentrations across groups is shown in Figure 8. There was a significant concentration effect for PLLA-PEG 1000 for all combinations of concentrations in all genes tested with $p<0.005$, except for Collagen type X, where 20 vs. 25% and 25 vs. 30% had $p=0.286$ and $p=0.022$, respectively, Collagen type II, where 25 vs. 30% had $p=0.022$, and MMP13 where 25% vs. 30% had $p > 0.5$. As for PDLLA-PEG 1000, there were significant concentration effects for 25% vs. 30% across all genes with $p<0.05$. For PDLLA-PEG 4000, a significant concentration effect existed only for 20% vs. 30% in Collagen type II with $p=0.045$. Overall, multivariate analysis of PDLLA-PEG 4000 vs PDLLA-PEG 1000 revealed no significant differences across all genes except MMP13, which has a $p=0.027$.

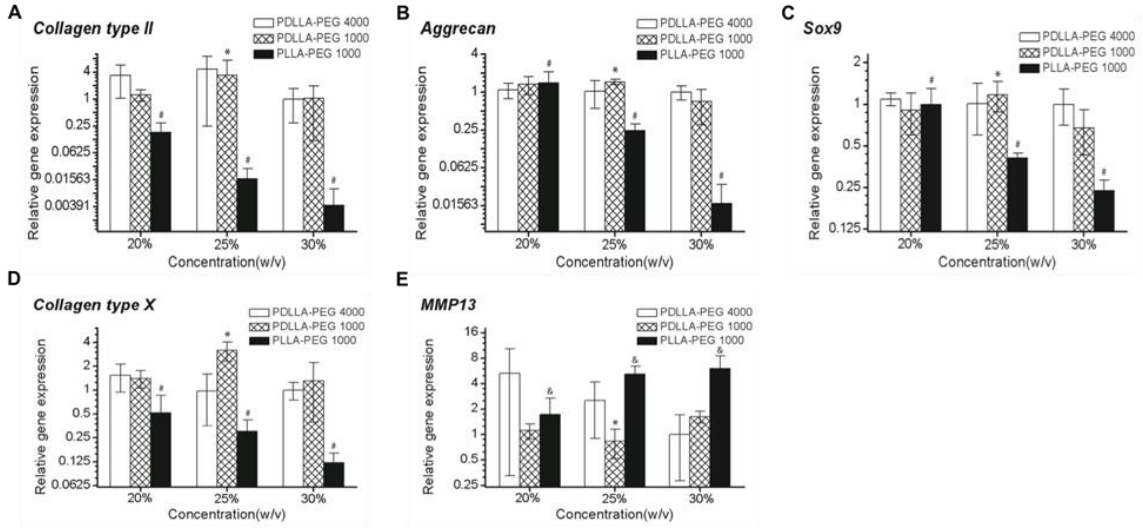

*Figure 8. Real-time PCR analysis of gene expression in hBMSCs at day 28 in constructs. Relative gene expression levels of (A) Collagen type II, (B) Aggrecan, (C) Sox9, (D) Collagen type X, (E) MMP13 normalized to cell gene expression in PDLLA-PEG 4000 at 30% w/v material concentration. Overall, two-way independent MANOVA analysis of PDLLA-PEG 4000 vs. 1000 revealed no significant differences across all genes except MMP13 ($p=0.027$). *$p<0.05$ for PDLLA-PEG 1000 25% vs. 30%. #$p< 0.005$ between all concentrations for PLLA-PEG 1000 except Collagen type X, where 20% vs. 25% had $p=0.286$. & $p<0.005$ between 20% vs. 25% and 30%.*

Histological analysis of ECM production using Safranin O/fast green and Alcian Blue/fast red staining is shown in Figures 3 and 4. Uniform strong GAG deposition is seen across all concentrations in the PDLLA-PEG 4000 group with decreasing staining as concentration of material is increased (Figures 9A-C, 10A-C). PDLLA-PEG 1000 group exhibits a similar trend of strong staining decreasing as concentration of the material increases (Figures 9D-F, 10D-F). In the PLLA-PEG 1000 group, cells exhibit weak staining across all three concentrations (Figures 9G-I, 10G-I). Histological sections correlate well with gene expression data with PLLA-PEG 1000 scaffolds showing significant differences in gene expression levels.

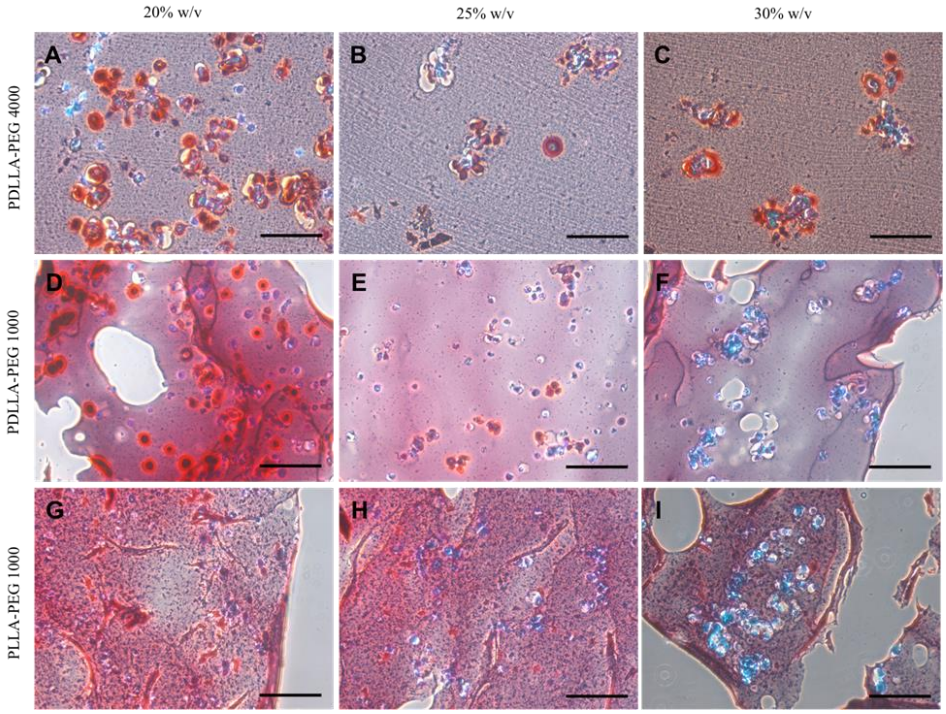

Figure 9. Glycosaminoglycan content in hBMSC-encapsulated constructs visualized by Safranin O/fast green staining at day 28. (A-C) Staining of PDLLA-PEG 4000 group. Staining decreases as concentration increases. (D-F) Staining of PDLLA-PEG 1000 group. Similar to PDLLA-PEG 4000. (G-I) Staining of PLLA-PEG 1000. Staining is weak in all groups. Scale bar = 150 μm.

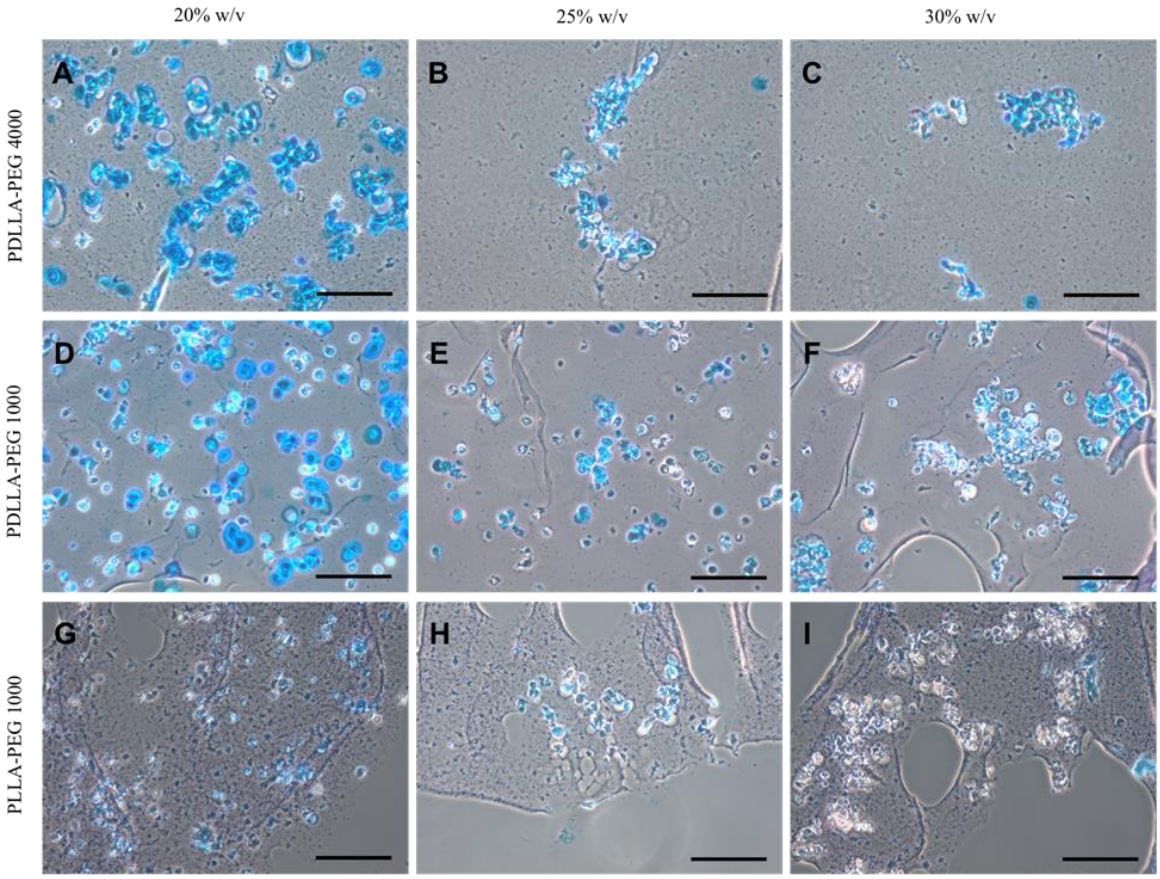

Figure 10. Glycosaminoglycan content in hBMSC-encapsulated constructs visualized by Alcian blue/fast green staining at day 28. (A-C) Staining of PDLLA-PEG 4000 group. Staining decreases as concentration increases. (D-F) Staining of PDLLA-PEG 1000 group. Similar to PDLLA-PEG 4000. (G-I) Staining of PLLA-PEG 1000. Staining is weak in all groups. Scale bar = 150 μm.

In summary, PDLLA-PEG 1000 will used for in vivo study.

4. For adipose stem cell (ASC) chondrogenesis, BMP-6 has been shown to significantly augment the matrix production. Therefore, we further included different amounts of BMP-6, from 1 μg/ml to 8 μg/ml, together with 2 μg/ml TGF-β3 and test the ASC chondrogenesis within these chondroinductive factors-encapsulated PDLL-PEG1000. We have finished all the culture and are performing the analysis. GAG assay result is shown in Figure 11. At low BMP-6 concentration (1 and 2 μg/ml), the addition of BMP-6 did not significantly enhance the cartilage matrix deposition. With the inclusion of 4 μg/ml BMP-6, rabbit ASC displayed enhanced chondrogenesis. Together with 2 μg/ml TGF-β3, 8 μg/ml BMP-6 stimulated maximum matrix production (around 50% more than 2 μg/ml TGF-β3 group). We are still working on the histological analysis. Based on current result, 2 μg/ml TGF- β3 combined with 8 μg/ml BMP-6 will be used for in vivo study.

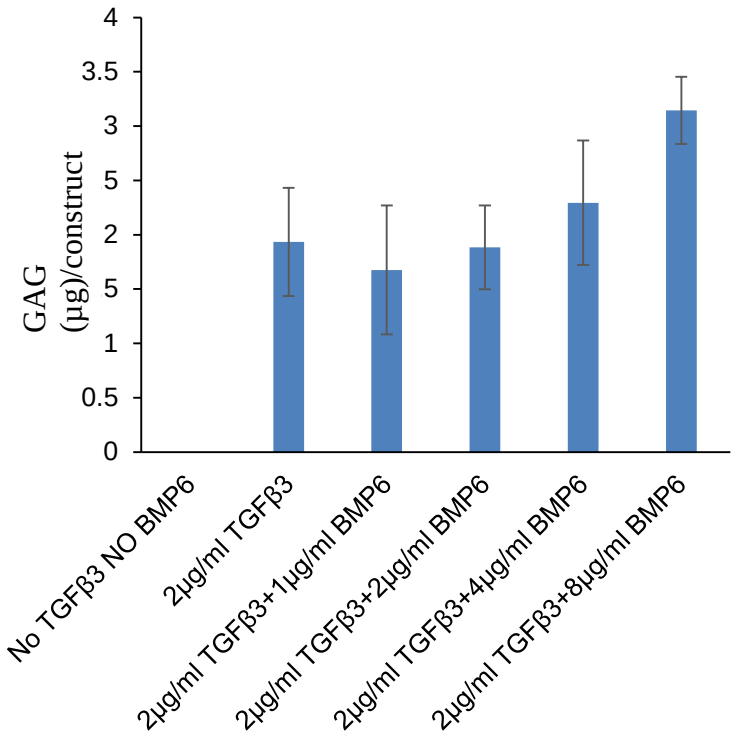

Figure 11. Total GAG assay

5. As reported before, we have successfully synthesized photocrosslinkable PCL as the scaffolds for bone formation. As shown in Figure 12, PCL scaffolds with different architectures, including condyle-like structures, have been fabricated through projection stereolithography

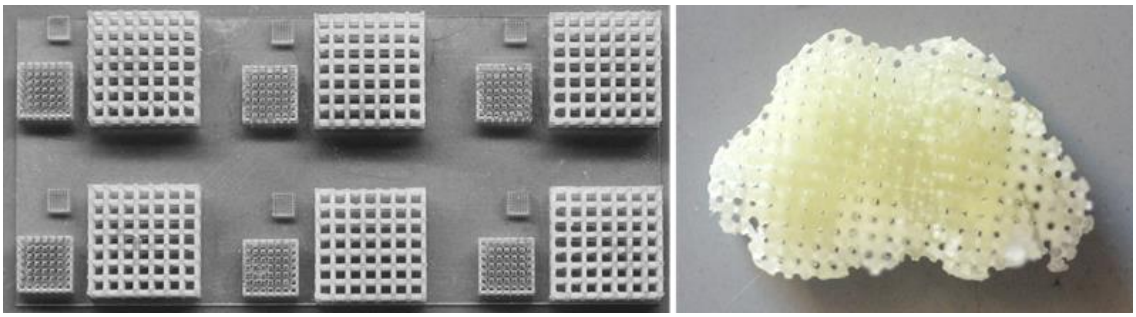

Figure 12, PCL scaffolds printed through projection stereolithography.

6. In our proposal, we will also conduct *in situ* osteogenesis within PCL scaffolds. In order to do so, we need deliver BMP-2 onto PCL scaffolds. Not like hydrogel, PCL scaffold is porous and hydrophobic thus not able to control the release of growth factors by themselves. We then coated the surface of PCL scaffold with heparin-DOPA, in which heparin is able to capture BMP-2 specifically and DOPA is used to bind PCL. By this way, we are able to load BMP-2 onto PCL scaffolds and utilize heparin to slowly release BMP-2.

To test our hypothesis, we soaked PCL scaffolds (Control) or heparin-DOPA coated PCL scaffolds (Heparin-DOPA) into BMP-2 solution and then seed stem cells into scaffolds. The constructs were then cultured in medium without supplements of BMP-2 protein. After 14 days culture, we collected RNA and conducted real time PCR.

As shown in Figure 13, we saw similar results. Cells cultured in Heparin-DOPA coated and BMP-2 loaded PCL scaffolds displayed significantly higher osteogenic gene expression, including ALP, BSP and RunX2. Such results suggested more BMP-2 was reserved in heparin-DOPA coated scaffolds and induced higher osteogenesis.

There conditions will be used for bone regeneration in animal study.

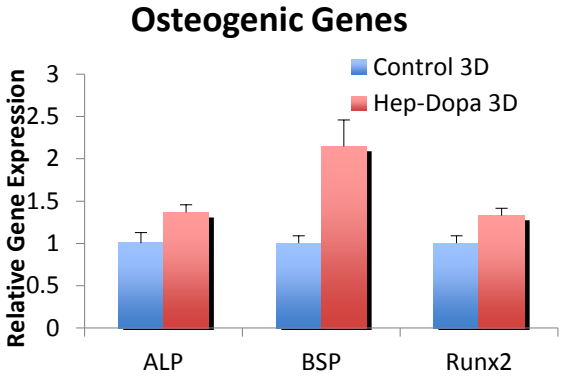

Figure 13. Relative gene expression of ASCs in PCL (Control 3D) or in PCL coated with Heparin-DOPA (Hep-Dopa 3D).

7. As shown in Figure 14, we are able to capture the structure of condyle in rabbit knee at different depth. Typically, it will take 8 hours to finish the scan. Using ScanIP software, we are able to generate a 3D models from original DICOM files (Figure 15).

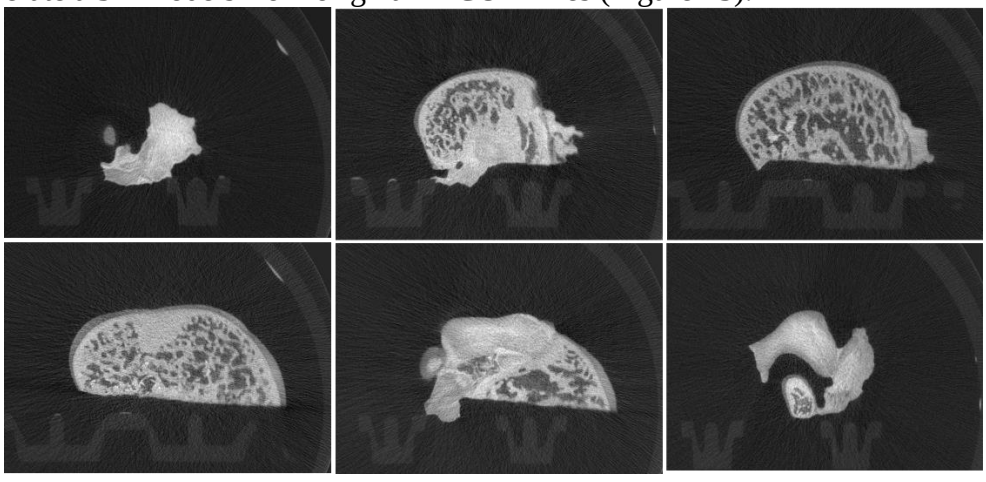

Figure 14. Original DICOM imaging after microCT scan.

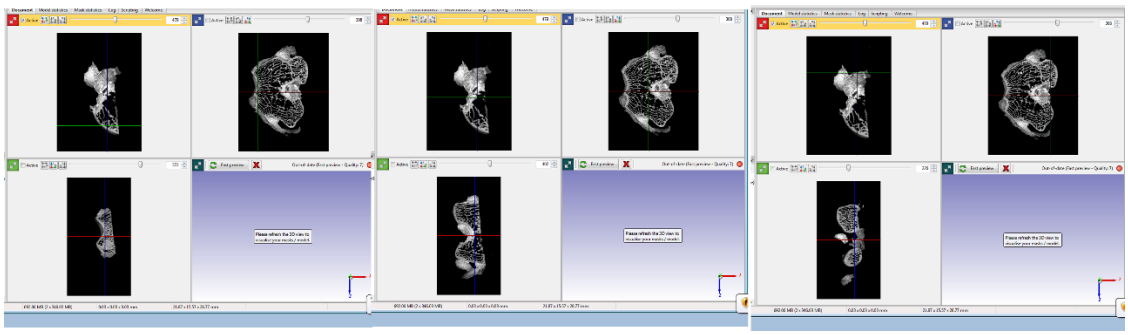

Figure 15. 3D printable models generated from original DICOM files

Furthermore, as shown in Figure 16, we are able to print PCL bone scaffold (will be used for in vivo study) using the uCT imaging as the template. We are currently establishing a procedure that is able to quickly introduce pores, with different pore size and internal architecture, into solid condyle scaffold. The final goal will be less than 30 minutes.

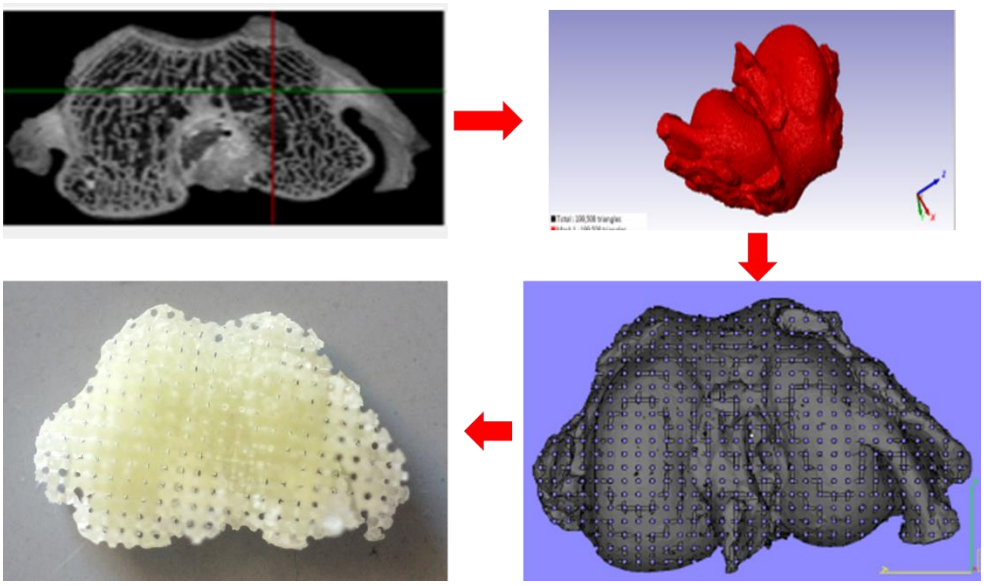

Figure 16. PCL bone scaffold produced from uCT imaging

To now, we have optimized cell density, growth factors concentration and scaffolds for in vivo study.

As proposed, we will test the regeneration in both healthy and OA animals. Since the creation of OA model will take more than 3 months. We are working to optimize the procedures.

8. Testing the response of encapsulated ASCs within the hydrogel after traumatic impact or in inflammatory condition in vitro. In preparation of the implanting the hydrogel into an iatrogenic or focal osteoarthritic defect, the effects of impacts on bovine chondrocyte hydrogel constructs in vitro were tested. To examine the molecular changes after impacts at 20% strain, bovine articular chondrocytes were isolated from bovine articular condyles, digested in type II collagenase at 37 °C overnight, and further encapsulated in methacrylated gelatin (mGL) and agarose VII (Ag)

constructs at $20x10^6$ cells/ml. Constructs were impacted at 20% strain (20% maximum displacement) prior to the culture in TGFβ3-supplemented chondrogenic medium for 7 days. Constructs were collected on day 1 for
live/dead assay; constructs for RT-PCR were analyzed on day 1, 3, and 7 (n=3~7 per trial, three independent trials); constructs for histological detection were also analyzed. On day 1 post-impact, live/dead staining showed some dead cells (red color) along the fissure in both mGL constructs and Ag constructs (Figure 10).

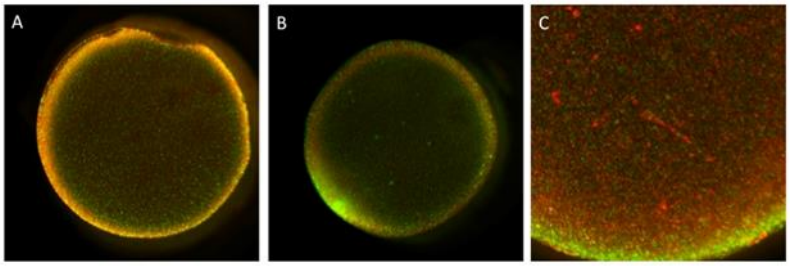

Figure 17. Live/dead staining of bovine chondrocyte mGL and Ag constructs impacted at 20% strain on day 1 compared to control. (A) Image of an impacted mGL construct (25X). (B) Image of an unimpacted control mGL construct (25X). (C) Image of an impacted Ag construct (67.5X).

In mGL hydrogel cultures, no difference between unimpacted (control) and impacted was observed in the gene expression of aggrecan and collagen type II; expression of COL10, MMP13, RUNX2, ADAMTS4 and ADAMTS5 was slightly but not significantly increased in impacted group at day 7 **(**Figure 18). However, the effect of impacts was not observed from histological analysis (Figure 19).

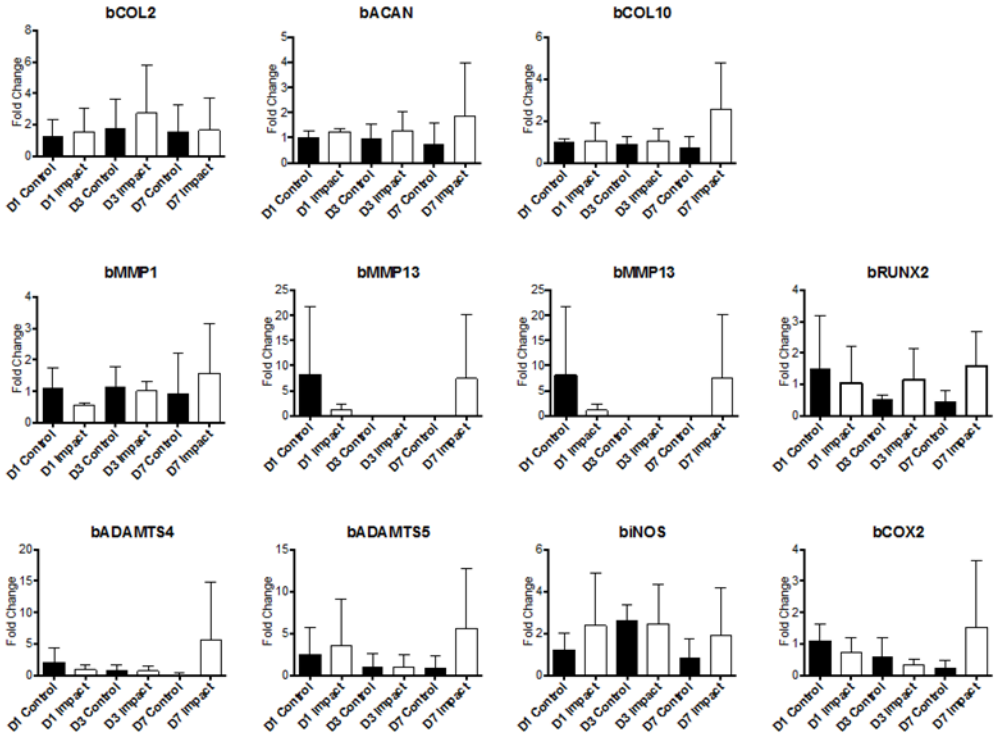

Figure 18. Gene expression of bovine chondrocyte mGL constructs impacted at 20% strain.

Agarose hydrogel cultures, gene expression of aggrecan and collagen type II increased on day 3 but no difference between unimpacted (control) and impacted was observed in agarose constructs (data not shown). On day 7, collagen type X down-regulated when MMP13, RUNX2 and ADAMTS4 upregulated in the impacted constructs, suggesting impact-induced matrix degradation starts on day 7 (data not shown). However, from histological analysis, cartilage deposition decreased in the umimpacted constructs during 7-day culture; staining of impacted construct on day 3 was surprisingly weak but we postulated this single construct was not impacted (Figure 19).

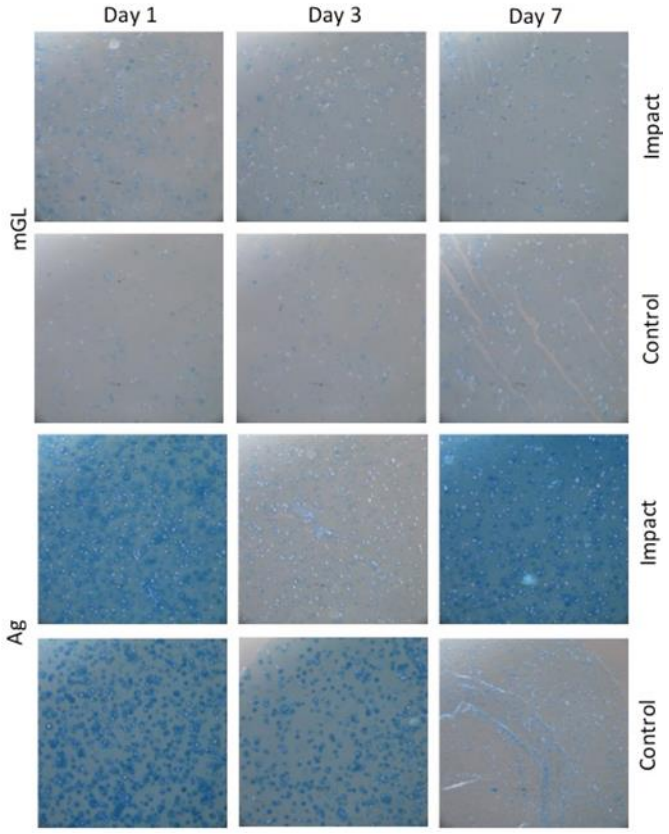

Figure 19. Histological analysis of bovine chondrocyte mGL and Ag constructs impacted at 20% strain.

9. Successful posterior approach to the rabbit femoral condyle with manual traumatic impact delivery We have previously reported on the use of a spring-loaded impactor to deliver traumatic impacts to articular surfaces and produce focal osteoarthritic defects. Next, we aimed to develop a posterior approach to the rabbit knee in order to position the implant in the weight-bearing region of the rabbit knee. We have previously used an anterior approach, which is only weight-bearing during extreme extension (energetic hopping). Tochigi et al have reported that the majority of the habitual contact region is accessible only by a posterior surgical approach. Thus, we explored the feasibility of a posterior approach combined with a manual delivery of traumatic impact using the spring-loaded impactor previously described.

Following anesthetization, 2-year old New Zealand White Rabbits underwent computed tomography imaging on the right knee using the FIDEX (Animage, Pleasanton, CA, USA) apparatus. Following a return to the operating room and appropriate aseptic preparation of the surgery area, the animal was then placed prone onto an elevated operating table with the operative leg secured in full extension (Figure 20A). The skin was prepped using chlorhexidine and betadiene scrubs, and the limb was draped using sterile towels. The initial incision was positioned on the medial aspect of the lower limb from the mid-thigh to the proximal third of the leg. Meticulous care was taken to avoid significant bleeding from the superficial fat and subcutaneous vessels using suture ligation with 4-0 polydioxanone (PDS II, Ethicon, Johnson and Johnson, New Brunswick, NJ, USA) or a handheld cautery. The dissection was extended into the interval between the semitendinosus and the medial head of the gastrocnemius via blunt dissection. At this time a small incision was made into the muscle belly of the proximal third of the medial head of the gastrocnemius, avoiding the neurovascular bundle and the muscle's tendinous insertion. Blunt retraction through this incision revealed the posterior aspect of the knee capsule directly overlying the medial femoral condyle. A surgical arthrotomy was performed that revealed the medial femoral condyle from 120-180 degrees of its axis (Figure 20B). This has previously been associated with the weight-bearing region of the rabbit. Utilizing a sterile tip with a stabilizing silicone covering (Figure 20C), a controlled (estimated) 36 MPa impact was performed onto the revealed area of the medial femoral condyle (Figure 20D).

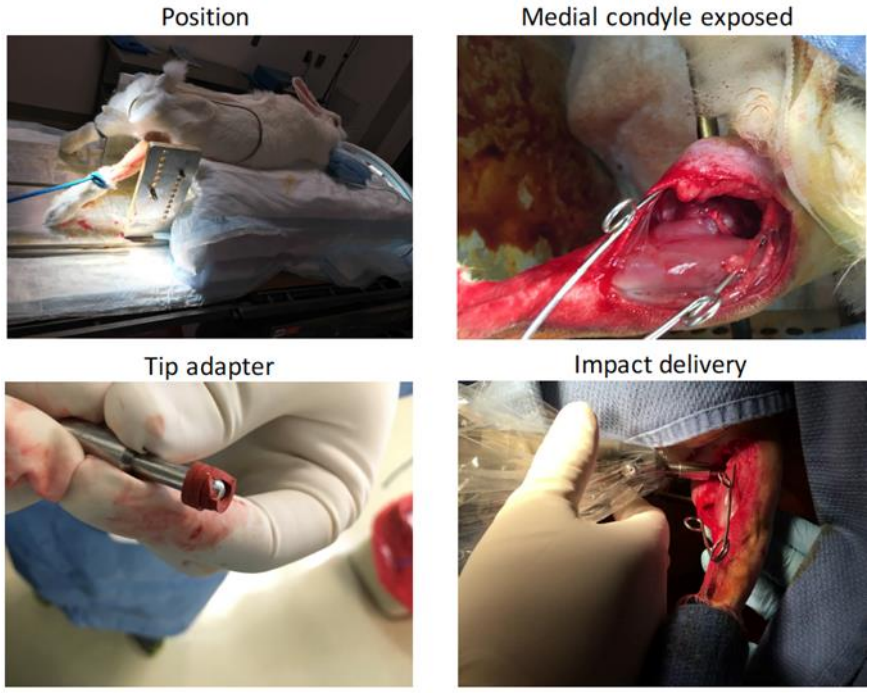

Figure 20: impact of posterior aspect of the medial femoral condyle follow posterior approach and exposure. (A) positioning of the patient (rabbit) for surgery prior to sterilization and draping; (B) exposure of the posterior aspect (weight-bearing) of the medial condyle. (C) preparation of the impact tip with a silicone sleeve to permit and maintain proper positioning; (D) position of the impactor prior to delivery of the traumatic impact

Following the impact, the muscle incision was repaired primarily using horizontal mattress 4-0 PDS suture. The empty space created by the blunt dissection was also closed primarily using 4-0 PDS suture. A subcutaneous running skin/subcuticular closure was performed using 4-0 PDS, followed by skin glue to promote primary healing and prevent animal access to the suture material. In situ impacts using the impact device were compared to those delivered to explanted cartilage plugs in a fixed arrangement. 400mV reading corresponds to 42 MPa over an estimated impact area 1.2mm in diameter (Figure 21).

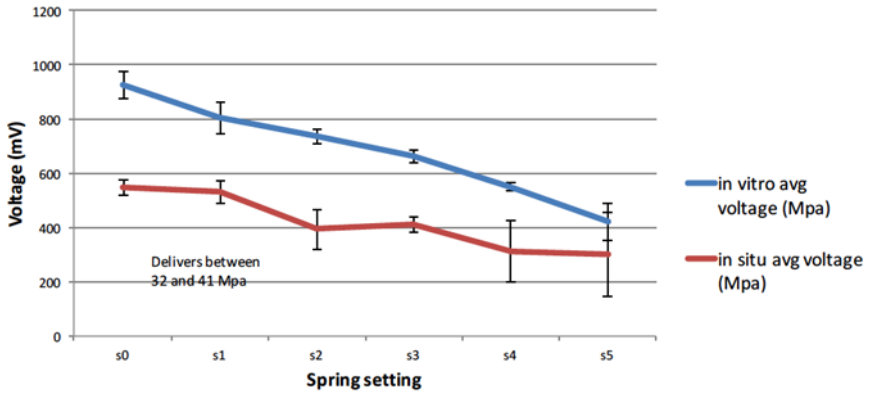

Figure 21: a comparison of the detected voltage recorded during the delivery of impacts with a clamped impactor on explanted bovine articular cartilage (blue) or a manually held impactor on cadaveric rabbit femoral condyles in situ (red).

The experiment, still on-going, involves impacted and sham operated animals harvested 1, 3 and 7 days after impact to assess the earliest changes in the cartilage after trauma and 4 and 12 weeks after impact to characterize the resultant focal osteoarthritic lesion.